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Full-Length Research Article

Sex-Specific Sub-Acute Toxicity and Immunomodulatory Profile of a Nigerian Polyherbal Supplement (Napherbs) In Albino Wistar Rats

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Summary: NaPherbs, a Nigerian-based polyherbal supplement compounded with Moringa oleifera, Vernonia amygdalina, Garcinia kola, etc. is consumed locally as an immune-boosting nutraceutical. In this study, we investigated its preliminary immune-boosting mechanisms and sub-acute toxicity profile in both male and female albino Wistar rats. A total of 40 Wistar rats (20 males and 20 females) were randomly allotted to 5 experimental groups (n=8, 4 males and 4 females); control, 125, 250, 500 and 1000 mg/kg/day of NaPherbs for 30 consecutive days. The animals were anaesthetized, and cardiac puncture was used to obtain serum for biochemical assays such as the serum lipid profile, immunoglobulin concentration, proinflammatory cytokines' concentration and liver function enzymes; and whole blood for haematological assessment. In vivo antioxidant assays were carried out also on the liver homogenates, while the liver and kidneys were examined histologically in both male and female animals. There was a dose-dependent increase in the neutrophil: leucocyte ratio in the female animals. NaPherbs did not pose any major cardiovascular risk across the groups, and there seemed to be some level of cardioprotection judging from the reduction in low-density lipoprotein cholesterol (LDL-c), triglyceride (TG) and total cholesterol (TC) observed in both sexes. The antioxidant indices assayed for were not significantly ($P<0.05$) affected by NaPherbs treatment, except for the catalase enzyme which was significantly increased in the female animals. The pro-inflammatory cytokines and immunoglobulins assayed for were not significantly affected by NaPherbs. Histological examination of the liver and kidneys showed that NaPherbs did not cause any major deleterious effect to these organs, except for mild infiltration of the sinusoids with inflammatory cells in the hepatocyte. NaPherbs showed a consistent profile of safety across the animals and doses considered in this study. However, further immunologic assays must be explored to validate its immune-boosting claims and more specific toxicity assays must be employed to focus more on neurotoxicity, nephrotoxicity and gonadotoxicity in both sexes.

Keywords*: NaPherbs, immunomodulatory, subacute toxicity, histopathology, lipid profile, neutrophil/leucocyte ratio (NLR)*

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INTRODUCTION

Natural product remediation remains a major part of mainstream medicine and not just an alternative therapy (Elkordy *et al*., 2021). Recently, the therapeutic role of nutraceuticals, such as functional foods and dietary supplements, in health care has been identified (Vignesh *et al*., 2024). This is mainly important as prophylactics and therapeutics, especially in resource-limited settings (Puri *et al*., 2023). Nutraceuticals are derived from food and provide health benefits beyond basic nutrition, acting as preventive or therapeutic agents against various conditions (Vignesh *et al*., 2024). Research findings show that about 80% of Asians and Africans subscribe to natural product medicines (Okaiyeto & Oguntibeju, 2021). This is mainly due to their affordability, availability, accessibility and perceived safe profile. It is also believed that combining several functional foods into formulas could harness their individual efficacies and provide synergistic therapeutic mechanisms. In spite of these folkloric claims and beliefs, many of these supplements and formulas still lack scientific validations of efficacy, toxicological profile and particular interactions such as drug-herb interactions (Gamil *et al*., 2025). In addition to these, gender-specific data, pediatric peculiarities and geriatric considerations are still far-fetched. For the purpose of standardization and safety, many developed countries exert some degree of regulation and strategies for monitoring these polyherbal supplements (Dubale *et al*., 2025). However, in many developing countries, several complementary and alternative medicine practitioners are not compliant with the WHO certification scheme to regulate the quality of these products. These explain the several divergent schools of thought and opinions on the use of medicinal products (Awodele *et al.,* 2013; Romero-García *et al.,* 2024).

NaPherbs is a Nigerian-based polyherbal formula primarily composed of edible seeds and plants. It is locally compounded in Kwara state, Nigeria, where it is being consumed as a medicinal supplement by both males and females. This formula became quite popular during the height of the COVID-19 pandemic, when it was packaged as teabags and steeped in hot water. This popularity leveraged the folkloric claim that it is an immune-boosting supplement that could help fight viral infections.

Therefore, we set out to explore a preliminary immunomodulatory and subacute toxicity profile of NaPherbs in both male and female animals, to establish or provide further insight into its efficacy and safety in both sexes.

MATERIALS AND METHOD

Animals: Eighty albino Wistar rats (40 males and 40 females) were used in this study. Rats received pelletized rat chow and water *ad libitum*.

Chemicals: Ketamine (anesthetic agent; 40 mg/kg/body weight) and ethanol were purchased from *Sigma Aldrich*, USA.

Preparation of NaPherbs extract: As previously reported by Afolabi *et al*. (2023), briefly, NaPherbs is a polyherbal nutraceutical containing: *Ocimum gratissimum* (5% w/w); *Garcinia kola* (5% w/w); *Zingiber officianale* (5% w/w); *Vernonia amygdalina* (5% w/w) and leaves of *Moringa oleifera* (80% w/w). NaPherbs (500 g) was successively macerated in 2.5 L of ethanol for 72 h, filtered with Whatman number 1 filter paper. The residue was reconstituted in fresh ethanol (2.5 L) twice, to increase the percentage yield. The filtrate was concentrated using a rotary evaporator at 40°C (BUCHI Rotavapor® Model R-215, Switzerland) with the vacuum Model V-801 EasyVac® Switzerland.

Experimental design: Procedures involving animal handling followed the guidelines published by the National Institute of Health (NIH, 1996) and the University of Ilorin Ethical Review Committee (UIERC) with approval number: UERC/ASN/2020/2034. The rats were randomly divided into 5 groups of 8 animals each (4 males and 4 females), control (distilled water); 125 mg/kg/day of NaPherbs; 250 mg/kg/day of NaPherbs; 500 mg/kg/day of NaPherbs and 1000 mg/kg/day of NaPherbs. The animals received oral administration of the corresponding dose for 30 days and weekly body weight was measured.

Hematological assays: After 30 days of treatment with NaPherbs, the rats were anaesthetized by an intraperitoneal administration of 40 mg/kg ketamine and blood was drawn by cardiac puncture. A portion of the blood sample was centrifuged for 20 min at 3000 g, and the serum was harvested and frozen for serum biochemical assay. The remaining portion of blood was put into a EDTA tube for hematological assays. Parameters such as WBC, % neutrophils, % platelets, etc were assayed for according to methods described by Kale *et al*. (2019) using using an automated hematology analyzer from *HORIBA Pentra™ XL 80,* USA.

Serum biochemical parameters: Serum lipid profile [triglycerides (Trig), total cholesterol (TC), very low-density lipoprotein (VLDL-c), high-density lipoprotein (HDL-c), and low-density lipoprotein (LDL-c] were analyzed using commercial kits obtained from *Randox Laboratories Ltd* (Crumlin, UK), with compliance to the manufacturers protocol. Serum IL-6, IL-10 and TNF-α were estimated by the method of Yener *et al*. (2015) using ELISA kits obtained from Sigma-Aldrich®, Saint Louis, MO, USA. Liver function enzymes including alanine transaminase (ALT), aspartate transferase (AST), and bilirubin levels were evaluated using the Beckman Coulter LH 780 analyzer.

Liver antioxidant and MDA levels: According to the methods described by Afolabi *et al*. (2023), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) activities were carried out on the liver homogenate post-30 days treatment protocol. Analytical kits obtained from *MyBiosource Inc.* (San Diego, USA) were used for the assay. Malonaldehyde (a marker of lipid peroxidation) levels were estimated using analytical kits obtained from *MyBiosource Inc.* (San Diego, USA) and *Elab Sciences* (Texas, USA) respectively, and following the manufacturer's protocol (Afolabi *et al*., 2023).

Histological examination of the liver and kidneys:

Liver, and kidneys were fixed in 10% neutral buffered formalin as described earlier (Avwioro, 2010; Ojuade *et al*., 2021), Briefly, tissues were dried by successively passing them through ethanol at increasing concentrations of 70%, 80%, 90%, and 100%, and cleared in xylene before inserting in paraffin. Sections of 5 µm of tissues from liver and kidneys were dewaxed and stained with hematoxylin-eosin (H&E), placed on charged microscope slides (Asala *et al*., 2021) for histological examination and under a low and high-powered field of Carl Zeiss binocular microscope (New York Microscope Company, NY, USA). IC-3 mounted camera was used for photographing the microscopic lesion as described by *Akanbi et al*. (2021).

RESULTS

Effect of NaPherbs on body weight and organosomatic index: NaPherbs did not cause any significant ($P<0.05$) change in the body weight during the 30 days experimental phase of the study. Also, the organosomatic indices for the liver, kidneys, heart, spleen and pancreas was not significantly ($P<0.05$) altered. This is shown in figures 1A and 1B.

Effect of NaPherbs on hematological parameters: NaPherbs caused a significant ($P<0.05$) increase in the percentage of neutrophil (Figure 2A), coupled with a decrease in the percentage of leucocyte (Figure 3B), leading to a dose-dependent increase in the neutrophil: leucocyte ratio in the female animals. This was not observed in the male animals, as there was no significant ($P<0.05$) alteration in these indices. Platelet count was also significantly ($P<0.05$) increased in both male and female animals at 500 and 1000 mg/kg, respectively (Figure 3A).

There was a significant ($P<0.05$), dose-dependent increase in the concentrations of both the mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) in the female animals. This is shown in Figures 3C and 3D.

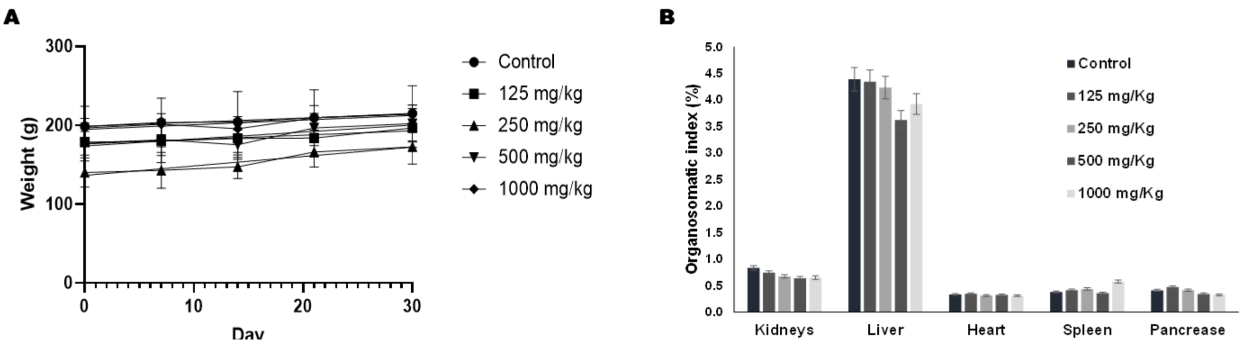

Figure 1.
Effect of NaPherbs on body weight and organosomatic index

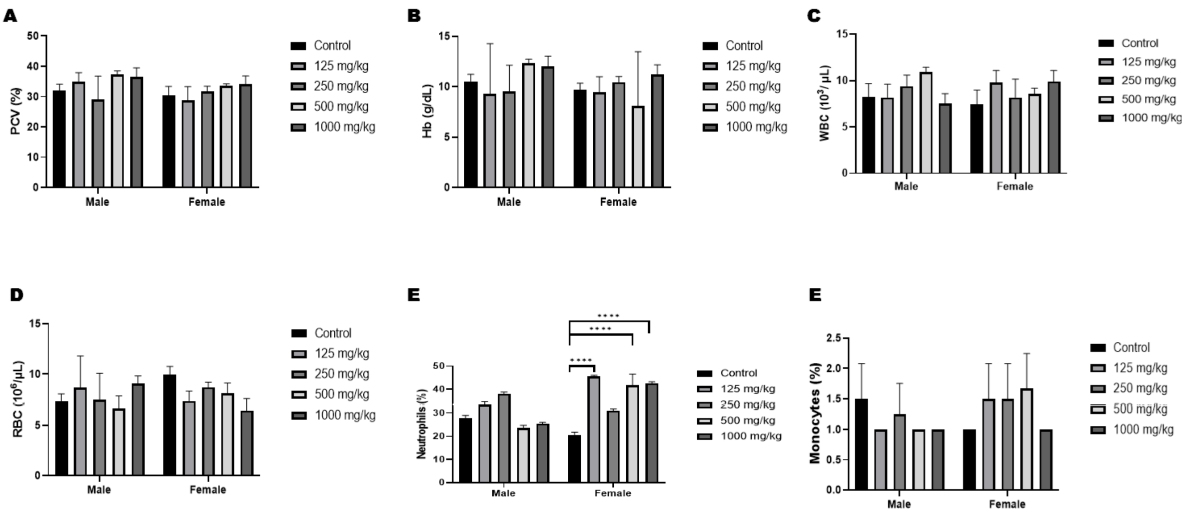

Figure 2.
Effect of NaPherbs on hematological parameters

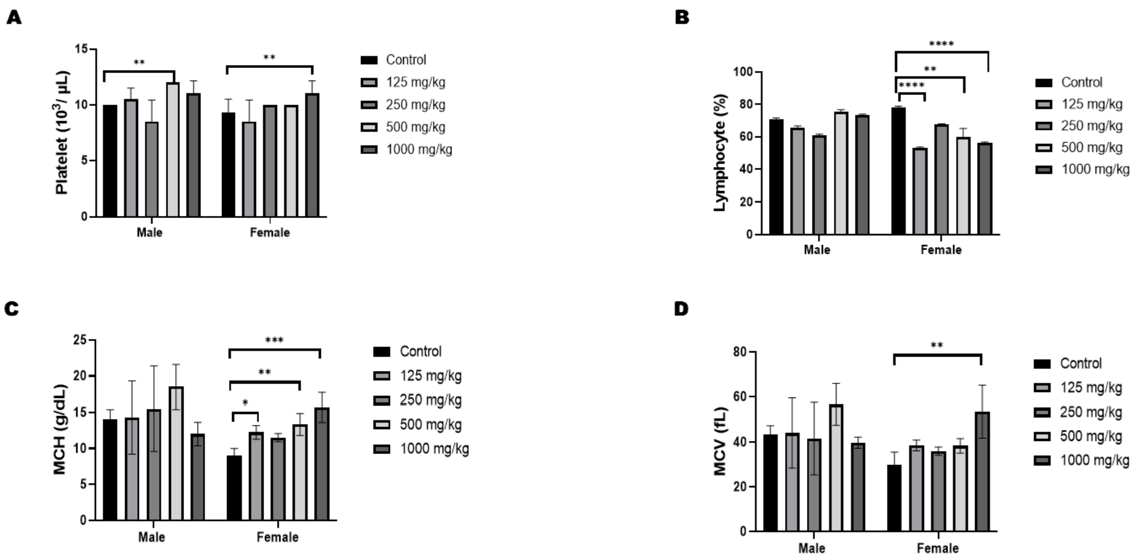

Figure 3.
Effect of NaPherbs on hematological parameters

Effect of NaPherbs on serum biochemical parameters: Considering the serum lipid profile, NaPherbs caused a significant ($P<0.05$) decrease in triglyceride (TG) and total cholesterol (TC) in the female and male animals, respectively (Figures 4A and 4B), when compared with the control group. There was also a significant ($P<0.05$) reduction in the concentration of the low-density lipoprotein cholesterol (LDL-c) in both male and female animals (Figure 4D) when compared with the control. NaPherbs caused a significant reduction in very low-density lipoprotein cholesterol (VLDL-c) concentration in the female group only at a dose of 500 mg/kg. Evaluating the effect of NaPherbs on immunoglobulins G, M and A; tumor necrotic factor-alpha (TNF-α) and interleukin 6 (IL-6), there was no significant ($P<0.05$) difference in the concentration of these immunoglobulins and proinflammatory cytokines when compared with the control (Figures 5A-5D).

Effect of NaPherbs on Liver antioxidant enzymes and liver function enzymes: Superoxide dismutase (SOD), reduced glutathione (GSH), and malondialdehyde (MDA) levels were not significantly ($P<0.05$) altered by exposure to NaPherbs in both male and female animals. The concentration of catalase was significantly ($P<0.05$) increased in the female animal group at the 250 mg/kg group, although this was not dose-dependent (Figures 6A-6D). The concentration of liver function enzymes, alanine transaminase (ALT), alkaline phosphatase (ALP), aspartate transaminase (AST) and serum urea were not significantly ($P<0.05$) affected by NaPherbs treatment in both male and female animal groups (Figure 7A-7D).

Effect of NaPherbs on the histoarchitecture of the liver and kidneys: Histological examination of the liver after 30 days of administration of NaPherbs showed in the male control, severe cytoplasmic fat infiltration with severe hepatic steatosis (Figure 8A), while the female control had a normal hepatocyte morphology, with sinusoids mildly infiltrated with inflammatory cells (Figure 8F). These morphological defects were not observed in the groups treated with NaPherbs, particularly in the male group, where hepatocyte morphology appeared normal, with only very mild infiltration of inflammatory cells (Plate 1B-E).

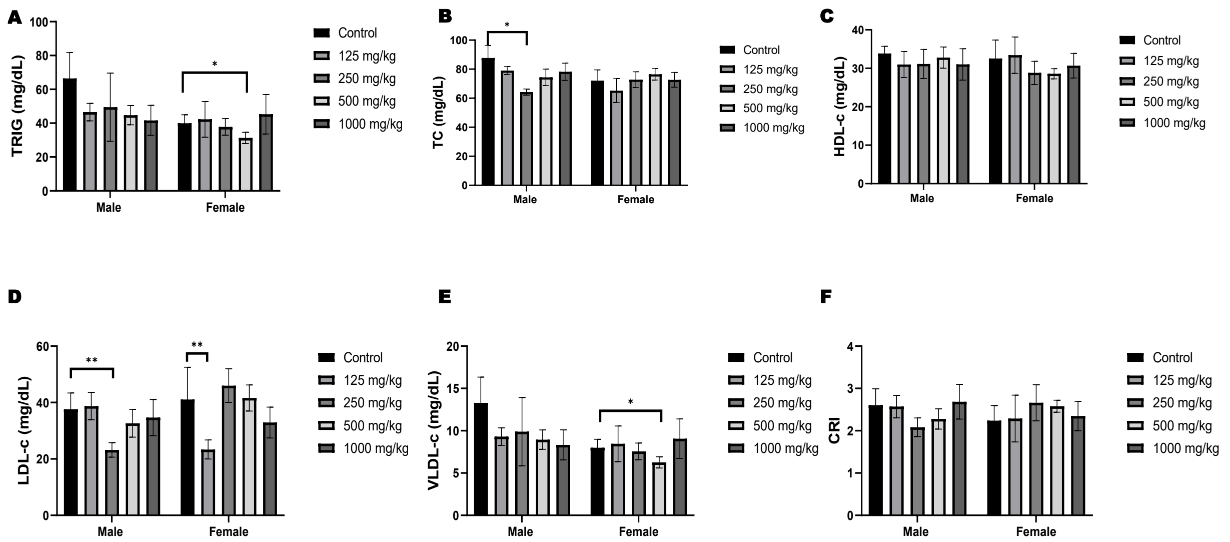

Figure 4.
Effect of NaPherbs on serum lipid profile

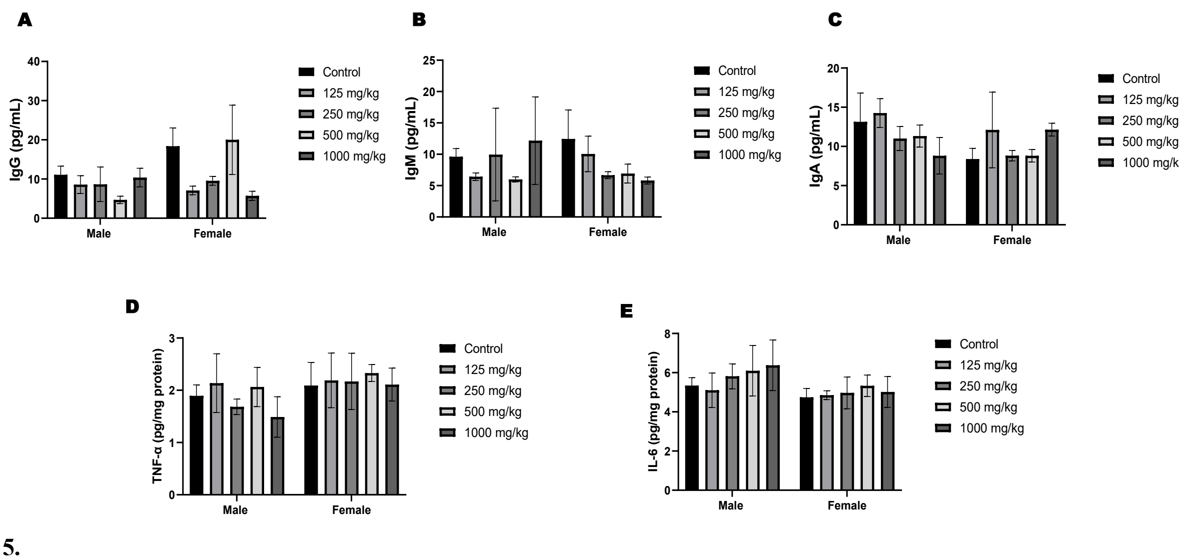

Figure 5.
Effect of NaPherbs on immunoglobulins and pro-inflammatory cytokines

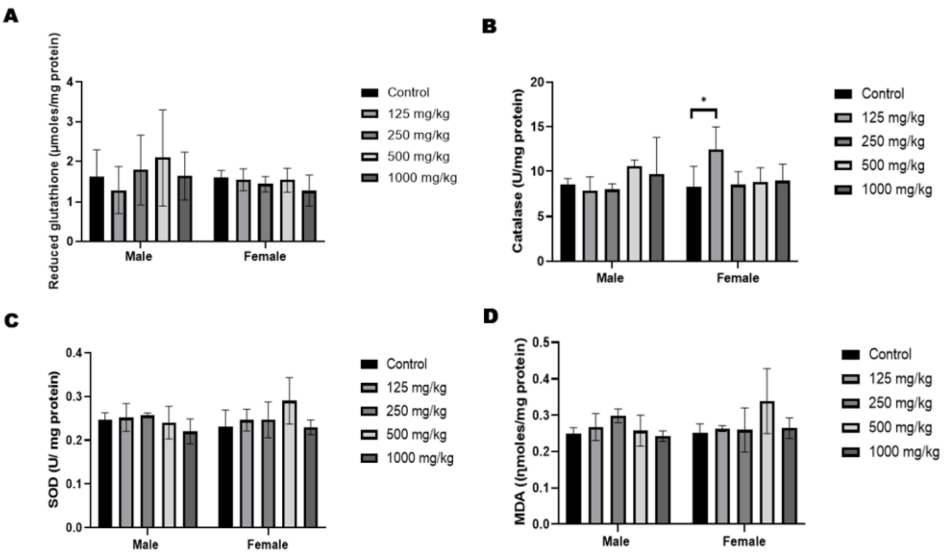

Figure 6.
Effect of NaPherbs on liver antioxidant enzymes and malondialdehyde levels

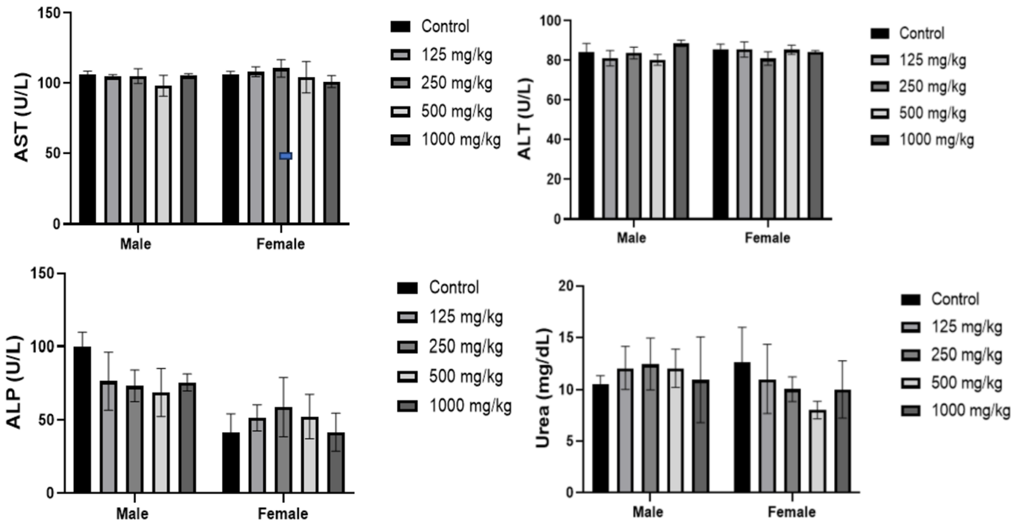

Figure 7.
Effect of NaPherbs on liver antioxidant enzymes and liver function enzymes

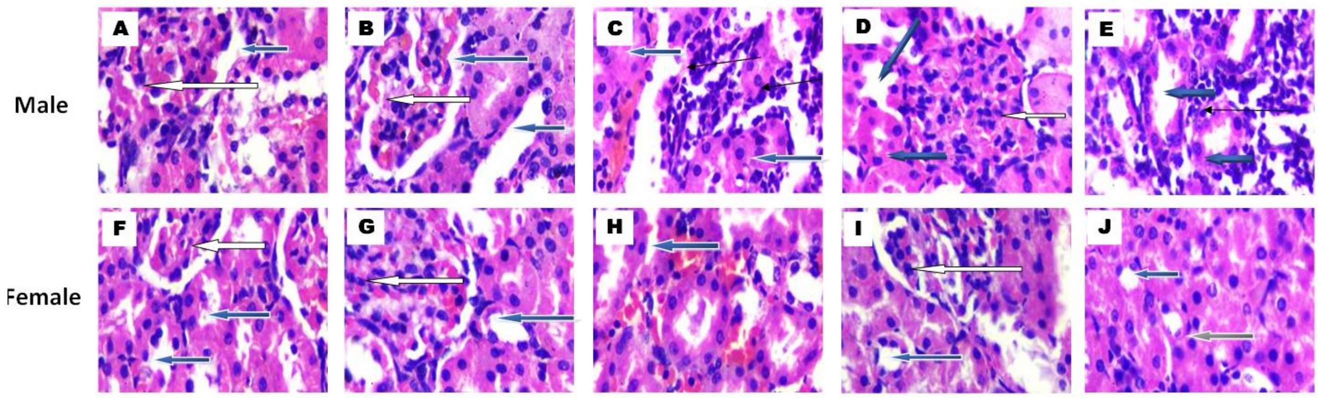

Plate 1
Histology of the liver (A) the morphology of the hepatocytes show severe cytoplasmic fat infiltration – severe hepatic steatosis (blue arrow), (B) the morphology of the hepatocytes show mild cytoplasmic vacuolation (blue arrow), the sinusoids appear normal and not infiltrated (slender arrow), (C) the morphology of several hepatocytes appear normal (blue arrow), few necrotized liver cells are seen (green arrow)the sinusoids appear normal and not infiltrated (the morphology of the hepatocytes appear normal (blue arrow), the sinusoids appear mildly infiltrated by inflammatory cells(slender arrow)slender arrow), (D-E) the morphology of the hepatocytes appear normal (blue arrow), the sinusoids appear mildly infiltrated by inflammatory cells(slender arrow); (F) the morphology of the hepatocyte appear normal (blue arrow), the sinusoids are mildly infiltrated by inflammatory cells (slender arrow); (G) the morphology of the hepatocytes appear midly degenerated with (blue arrow), the sinusoids appear mildly infiltrated (slender arrow); (H-I) the morphology of the hepatocytes appear normal (blue arrow), the sinusoids show very mild infiltration of inflammatory cells (slender arrow); (J) the morphology of the hepatocytes appear normal (blue arrow). Magnification: 400 X H & E.

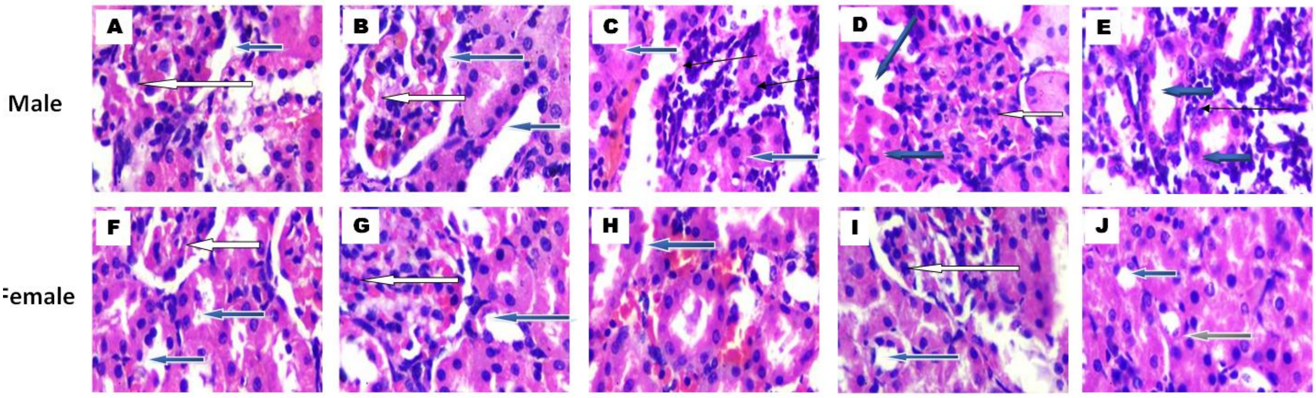

Plate 2.
Histology of the kidney showing, (A,F) the renal cortex with normal glomeruli and normal mesangial cells and capsular spaces (white arrow), the renal tubules appear normal (blue arrow), the interstitial spaces appear normal (slender arrow); (B,G) the renal cortex with normal glomeruli and normal mesangial cells and capsular spaces (white arrow), the renal tubules appear normal (blue arrow); (C) the renal cortex with normal glomeruli and normal mesangial cells and capsular spaces (white arrow),some the renal tubules appear collapsed and show loss of luminal spaces (blue arrow), the interstitial spaces appear moderately infiltrated by inflammatory cells (slender arrow); (D) the renal cortex with normal glomeruli and normal mesangial cells and capsular spaces (white arrow), the renal tubules appear normal (blue arrow), the interstitial spaces appear normal (slender arrow); (E) the renal cortex with normal glomeruli and normal mesangial cells and capsular spaces (white arrow), the renal tubules appear normal (blue arrow), the interstitial spaces show areas of mild to moderate infiltration of inflammatory cells(slender arrow); (H) the renal cortex with normal glomeruli and normal mesangial cells. The renal tubules appear normal (blue arrow); (I) the renal cortex with normal glomeruli and normal mesangial cells and capsular spaces (white arrow), the renal tubules appear normal (blue arrow); (J) some of the renal tubules appear normal (blue arrow) while few others lack lumen (green arrow). Magnification: 400 X H & E

In the kidneys, there was no major difference in morphological presentation between the control and treatment groups in both male and female animals. The renal cortex showed normal glomeruli, normal mesangial cells and capsular spaces. The renal tubules appear normal (blue arrow), and the interstitial spaces also appear normal (Plate 2A-9J).

DISCUSSION

Polyherbal supplements combining multiple herbs have become widely used as adjuvant therapies in Asia, Africa, and parts of the Americas (Moussavi *et al*., 2024). This is due to the believe that these formulations could work via synergistic mechanisms to improve efficacy, while ameliorating toxicities. NaPherbs is a locally consumed immune-boosting polyherbal supplement in Kwara state, Nigeria. It is packaged in the form of teabags and prepared via hot water extraction. It is rich in several phytomedicines including eucalyptol, alpha-pinene and gamma-terpinene and thus has gained relevance against several viral infections, including common cold (Afolabi *et al*., 2023). However, there are no established efficacy studies to validate these claims scientifically. Gender-based efficacy and safety testing is needed in order to improve acceptance, standardize the product, understand the underlying mechanism of efficacy and toxicity (Kale *et al*., 2019). This study explores the sub-acute safety profile and preliminary immune-modulatory indices of a Nigerian immunomodulatory polyherbal supplement (NaPherbs) in both male and female albino Wistar rats.

Effect of NaPherbs on hematological parameters such as neutrophil count, % PCV, WBC, % lymphocyte, etc., was evaluated. There was no significant alteration across the parameters in the male animals, except for the platelet count which was significantly increased in the 500 mg/Kg group. However, in the female group, there was a significant increase in neutrophil percentage across the groups, coupled with a dose-dependent reduction in the percentage of lymphocytes. This translates to a dose dose-dependent increase in the neutrophil/lymphocyte ratio (NLR) in the female animals. Neutrophils and lymphocytes are white blood cells responsible for combating infections (Barbalato & Pillarisetty 2022). Neutrophils are the body's first line of defence in the innate immune system, activated to contain most fungi and bacterial infections (Zhang *et al*., 2024). The lymphocytes govern the adaptive immune mechanisms which include T cells and B cells. It is characterized by a robust, targeted and specific response via release of antibodies to fight viruses and build long-term immunity (Sharma and Sharma 2022). A high NLR suggests an inflammatory response, physical stress and infection and this is indicative also of an underlying disease condition or pathological process. It has also been identified as an independent risk factor for onset of chronic kidney disease (Guo *et al*., 2022; Chmielewski *et al*., 2025). The mean corpuscular volume (MCV) is an indicator of the volume of RBC, while the MCH or mean corpuscular hemoglobin is the average mass of Hb/RBC in the blood sample. In the female animals, these values increased, especially at 1000mg/kg group. These could be as a result of an increase in reticulocytes (immature RBCs) formation. Also, this may be due to increased erythropoiesis induced by the polyherbal supplement. (Mitruka and Rawnsley, 1977; Adeyemo-Salami, and Ewuola, 2015).

NaPherbs is propagated as an immune-boosting supplement which became quite popular during the COVD-19 pandemic. However, these claims were largely folkloric with no scientific validation. We thought to check its effect on some of the major immunoglobulins. Immunoglobulins G, M and A have established antiviral activity which forms an integral part of the humoral immune network (Klingler *et al*., 2021; Patel & Jialal, 2023). Several mechanisms are

featured in humoral immunity, these include, preventing virus-host cell interactions; identifying viral antigens on virus-infected cells producing antibody-dependent cytotoxic cells (ADCC). IgG antibodies plays a major role in antiviral activity in the serum, while IgA is saddled with mucosal surface immunity (Klimpel 1996). There was no significant alteration in the concentrations of these immunoglobulins in both male and female animals after a sub-acute exposure to NaPherbs.
Oxidative stress underlies several disease conditions such as cancer, neurodegenerative disorders, cardiovascular disorders, etc. This is majorly caused by an imbalance between free radical production and the ability of the body's antioxidant enzymes to mop them up (Chaudhary *et al*., 2023). The liver being the major metabolizing organ is very important for total well-being and detoxification of xenobiotics or other harmful metabolites (Wang *et al*., 2025. Free radicals such as the reactive oxygen species (ROS) and reactive nitrogen species (RNS) have a single unpaired electron, making them highly reactive (Chandimali *et al*., 2025) and toxic to the hepatocytes. These free radicals, if not mopped up by antioxidants such as superoxide dismutase, glutathione peroxidase, catalase, etc., are injurious to organs, tissues and the entire biological system (Chandimali *et al*., 2025). NaPherbs did not majorly distort this well-ordered homeostasis mechanism between pro-oxidant and antioxidant factors in the liver. The product of lipid peroxidation, malondialdehyde levels in the liver was not also significantly affected across the groups in both male and female animals. However, there was a significant increase in liver catalase activity in the female group, which was not dose-dependent. Catalase enzyme is involved with the neutralization or mop up of hydrogen peroxide (H_2O_2), a potent oxidant to H_2O and O_2. An increase in catalase level suggests an active antioxidant defense mechanism against hydrogen peroxide, which is a potent oxidant, therefore protecting the tissues from damage as a result of oxidative stress (Nandi *et al*., 2019; Anwar *et al*., 2024).

Since NaPherbs is an acclaimed immune-boosting formula, asides assaying for immunoglobulins, we also explored its effect on some specific inflammatory mediators. A chronic inflammatory response underlies several disease conditions and the mechanism of action of several immunomodulatory drugs have been linked to suppressing inflammatory mediations (Zhao *et al*., 2021; Soares *et al*., 2023). We checked the effect the polyherbal formula on serum concentrations of tumor necrotic factor-alpha (TNF-α) and interleukin-6 (IL-6). There was no statistically significant alteration in the serum concentrations of these pro-inflammatory cytokines across the groups and sex. This suggests that the polyherbal formula might not act via inflammatory mediation to carry out its mechanism of immune-modulation. Increased production of IL-6 and TNF-α have been linked to chronic inflammation leading to tissue and organ damage (Bohl *et al*., 2024). Physiological concentrations of these cytokines play key functions in immunologic response to injury or infections. However, overproduction of these cytokines has been observed in disease conditions such as cancer, obesity, rheumatoid arthritis, etc. (Nie *et al*., 2025).

Abnormal lipid profile indices have been identified as a major risk factor in the onset and progression of atherosclerosis and several other cardiovascular disorders (Getie *et al*., 2025). Several epidemiological findings have shown that low levels of high-density lipoprotein cholesterol (HDL-c) and elevated levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-c) and very low-density lipoprotein cholesterol (VLDL-c) are indicators of increased risk of cardiovascular disorders (Dayimu *et al*., 2019; Taqiuddin *et al*., 2024). Our study showed that NaPherbs caused reduction on LDL-c in both male and female animals at 250 and 500 mg/kg respectively; while it reduced the concentration of TG and VLDL-c in female animals only and reduction of TC was only observed in the male animals at 500 mg/kg. There was no significant increase in the cardiovascular risk index (CRI) in both male and female animals. This suggests some level of cardioprotection in both sexes. Although, more specific cardiac protection models must be explored to establish these preliminary findings.

Serum concentrations of liver function enzymes such as alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) is indicative of the integrity of the liver, which is a major origan for drug/ xenobiotics metabolism and detoxification (Lee *et al*., 2012). While ALT is domiciled largely in the liver, AST can be found in other organs such as heart and skeletal muscles. This suggest that a high serum concentration of ALT in the serum coupled with a high concentration of AST is indicative of assault to the liver, either due to toxins, viral or bacterial infections (Lee *et al*., 2012). NaPherbs did not significantly alter the concentrations of these liver function enzymes in both male and female animals.

Evaluation of the histoarchitecture of organs particularly the liver and kidneys are a common tool for studying the integrity of the liver and kidneys (Bano and Najam, 2019). In the instance of the liver, a normal rat liver shows central vein and sinusoidal cords of the hepatocytes. However, the histoarchitecture of a diseased rat may reveal various abnormalities such as cellular distortion, fatty degeneration, inflammatory infiltration, etc. (Adeniyi *et al*., 2023). Histological examination of the liver in both male and female animal groups showed severe cytoplasmic fat infiltration and severe hepatic steatosis in the untreated (control) animals of both sexes. However, there was normal histoarchitecture of the liver in both the 500 and 1000 mg/Kg groups. These showed normal morphology of the hepatocytes with a mild infiltration of the sinusoids with inflammatory cells. For the kidneys, the control in both sexes showed the renal cortex with normal glomeruli, renal tubules, interstitial spaces and mesangial cells. This was also consistent with the treatment groups (250 – 500 mg/kg). NaPherbs did not significantly affect the histoarchitecture of the liver and kidneys in both sexes.

Putting these together, NaPherbs presents a safe profile across both animal sexes and doses considered in this study. However, there is need to further explore in more specifics, its effect on the integrity of the kidneys. This is due to the increased neutrophil: leucocyte ratio observed in the female animals, since this is an independent risk factor for the onset of chronic kidney disease. The immunomodulatory parameters assessed in this study did not indicate that NaPherbs significantly altered the immunological profile. There is a need to explore more specific immunologic assays at the cellular, tissue and organ levels. The authors suggest more specific assessments of organ toxicity, including neurotoxicity, nephrotoxicity, and gonadotoxicity.

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